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Cytotoxicity and Anticancer Studies of *Bacillus cereus* and *Bacillus pumilus* Metabolites Targeting Human Cancer Cells[1]

M. L. Vijaya Kumar[a], B. Thippeswamy[b], and P. Vasanth Raj[c]

[a] *Department of Microbiology, National College of Pharmacy*

[b] *Department of Microbiology, Kuvempu University, Shankarghatta-577451*

[c] *Department of Pharmaceutical Biotechnology, Manipal College of Pharmaceutical Sciences, Manipal, Karnataka, India*

e-mail: thippeswamyb205@gmail.com

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Abstract—The metabolites of bacteria *Bacillus cereus* and *Bacillus pumilus* isolated from soil samples in Shimoga region, Karnataka (India) were tested for cytotoxicity and anticancer properties. The various solvent extract fractions obtained from the metabolites of the two bacteria were tested for their cytotoxicity against normal human liver cell lines and 2 cancer cell lines by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay. The two fractions obtained from *B. cereus* showed high cytotoxicity. These two fractions were further screened for anticancer activity by nuclear staining studies and DNA fragmentation analysis. Both the fractions demonstrated significant activity by membrane blebbing during nuclear staining and caused the damage the DNA patterns during DNA fragmentation analysis. On the other hand, the metabolites of *B. pumilus* revealed toxic effect against cancer cells as well as normal ones.

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Microorganisms are one of the most important sources of new bioactive compounds such as antibiotics, immunosuppressants, antiparasitics, antitumor and hypocholesterolemic agents and enzyme inhibitors [1–4]. Many compounds which were first discovered as antibiotics failed in their later stages of development as antibiotics, but proved to be good antitumor, antimigraine, immunosuppressive and antiparasitic agents [1]. Each year millions of people are diagnosed worldwide with cancer, and more than half of these patients eventually die from this disease. Based on global cancer statistics published in the year 2011, 12.7 million cases of cancer were detected and 7.6 million cancer deaths in a year are reported [5]. Hepatocellular carcinoma is one of the most common malignancies throughout the world [6]. It is characterized by high incidence in hepatitis B virus associated liver diseases [7]. The present work is targeted against hepatocellular cancer cells. Conventional cancer treatments such as surgery, chemotherapy and radiotherapy often fail to achieve a complete cancer remission. Moreover, it has been widely recognized that radiotherapy or chemotherapy are likely to cause significant side effects. This fact has prompted the development of many new approaches for the treatment of cancer. One such example is the use of live, attenuated bacteria or their purified product. Microbial based therapy of cancer is one of the emerging cancer treatment modalities [8]. The use of bacteria in the regression of certain forms of cancer has been recognized for more than a century [9]. Important advances have been made to study and develop live bacteria or bacterial products such as proteins, enzymes, immunotoxins and secondary matabolites which specifically target cancer cells and cause tumor regression through growth inhibition, cell cycle arrests or apoptosis induction [10–13]. The other reason for using the microbial metabolites for anticancer activity was due to their high antioxidant activity by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) method which proves that they have free radical scavenging activity [14]. The aim of the study was to screen metabolites from *Bacillus cereus* and *Bacillus pumilus* for anticancer potential and to test their anticancer activity by nuclear staining studies and DNA fragmentation analysis.

MATERIALS AND METHODS

Reagents. All cultivation media components were purchased from Himedia (India). Chang liver cells (normal human liver cell lines), HepG2 (human cancerous liver cell lines) and Hep2 (human laryngeal epithelial carcinoma cell lines) were procured from the National Centre for Cell Sciences (NCCS) in Pune (India). Chemicals and solvents were purchased form Merck (Germany). Minimum essential medium (MEM), (3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (MTT), fetal calf serum, antibiotics and acridine orange were obtained from Sigma Chemicals Co.

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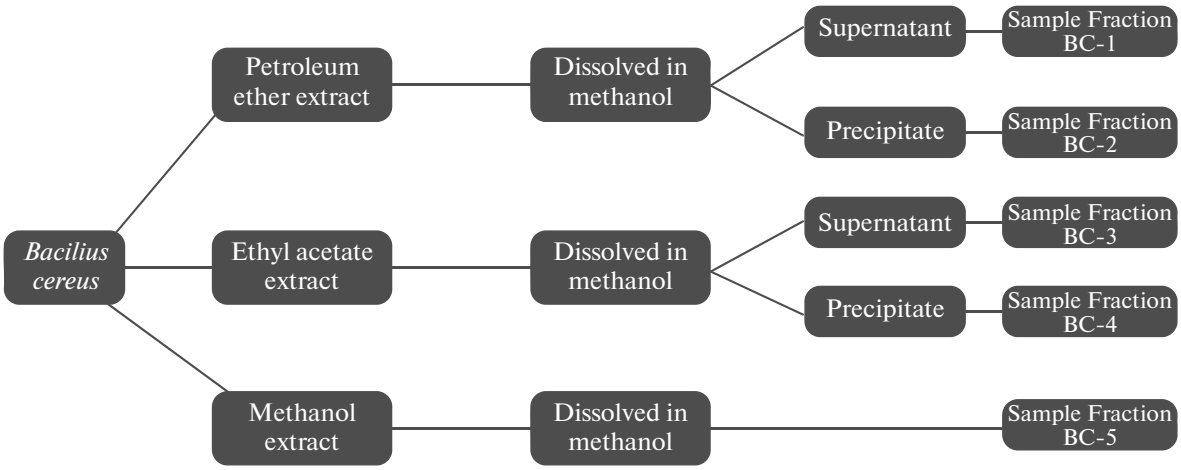

Fig. 1. Schematic diagram of preparation of *B. cereus* sample fractions.

(USA). Dimethyl sulfoxide (DMSO) was purchased from SD Fine Chemicals Ltd. (India).

Isolation and maintenance of bacterial cultures. Soil samples were collected from a hill station nearby Shimoga region, Karnataka (India). The microorganisms presented in the soil were screened for their antibiotic production potential by crowded plate technique [15]. Two bacteria with broad range of antibacterial activity were selected for further screening of their metabolites for anticancer activities. The two bacteria selected were identified as *Bacillus cereus* and *Bacillus pumilus* by lipid profile method [16] in National Institute of Oceanography, Kochi. The isolated pure cultures of *B. cereus* and *B. pumilus* were grown separately on nutrient agar medium [15] for 24 h at 35°C, and stored at 4°C until use.

Solvent extraction and preparation of samples. Both the bacteria were grown in nutrient broth medium by incubation at 35 ± 2°C for 3 days to obtain the metabolites. The broth was centrifuged at 2000 g for 20 min to remove the cells. The cell-free culture supernatants containing the metabolites were collected. After confirming antibacterial activity, the culture supernatants of both microorganisms were subjected to successive solvent extraction with petroleum ether, ethyl acetate or methanol (1 : 1) in a separating funnel. The obtained extracts were dried in separate plates to get thick, viscous compounds. These substances obtained from *B. cereus* were further separated and numbered as sample fractions from BC-1 to BC-5 as shown in the Fig. 1.

Similarly, sample fractions from BP-1 to BP-5 were obtained from *B. pumilus*. The sample fractions from BC-1 to BC-5 and from BP-1 to BP-5 were subjected to in vitro cytotoxicity studies by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

In vitro cytotoxicity studies. HepG2, Hep2 and Chang liver cell lines were used for the study. The sample fractions obtained as described in the above section were subjected to in vitro cytotoxicity studies by MTT assay [17].

The cell lines were cultured in MEM. The medium was supplied with 10% fetal calf serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL).

Cytotoxicity screening was carried out by determination of activity of mitochondrial succinate dehydrogenase by microculture tetrazolium assay [17].

The monolayer cell culture was trypsinized using combination of 0.2% trypsin and 0.5% EDTA. Cells were exposed to trypsin at 37°C for 3 min. The cell number was adjusted to 1.0×10^5 cells/mL using MEM containing 10% fetal calf serum [18]. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension containing approximately 10.000 cells was added. After 24 h, when a partial monolayer of cells was formed, the supernatant was flicked off, washed with 1 mM phosphate buffer (pH 7.3) and 100 μL of different *B. cereus* (BC-1 to BC-5) and *B. pumilus* (BP-1 to BP-5) sample fractions were added. The plates were incubated at 37°C for 3 days in 5% CO_2 atmosphere, then microscopic examination was made and observations were recorded for each 24 h. After 72 h, the drug solutions in the wells were discarded and 50 μL of MTT in MEM was added to each well. The plates were gently shaken and incubated for 3 h under the same conditions. The supernatant was removed, 50 μL of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader (Bio-Rad, USA) at 540 nm. The percentage growth inhibition values were calculated using the formula as given below.

$$\% \text{ growth inhibition} = 100 - \frac{(\text{mean } OD_{540} \text{ of individual test group})}{\text{mean } OD_{540} \text{ of control group}} \times 100.$$

Nuclear staining studies. Single cover slip was placed in each well of 6 wells microtitre plate. The monolayer HepG2 cell culture was trypsinized, the number of cells was adjusted to 50000 cells/mL and 2.5 mL of cell suspension was added drop by drop on cover slip in each well. After partial monolayer was formed, sample fractions of BC-1 and BC-3 prepared at different concentrations in maintenance medium cell culture were added. The control well contained only maintenance medium. The plates were incubated at 37°C in 5% CO_2 atmosphere. After overnight incubation, medium from wells was discarded and cells were washed with 10 mM phosphate buffer (pH 7.3, PBS). The cells were fixed with 1 mL of 90% methanol at 20°C for 20 min. The methanol was removed, replaced with acetone and kept for 10 s. After fixing, cells were washed with ice cold PBS for 2–3 times. The cells were incubated with PBS containing 1% BSA and 0.1% triton X-100 at 37°C for 30 min. Plate was washed with PBS for 2–3 times, 100 μL of 0.01% acridine orange in PBS (pH 7.4) was added and incubated at 37°C for 20 min. The cover slip was washed thrice with PBS and placed on the slide. The cover slips were observed under fluorescent microscope for any nuclear changes [10].

DNA fragmentation analysis. DNA was extracted from HepG2 cells. The liver cells were treated with 25 mM Tris–HCl buffer (pH 7.5) containing 0.5% SDS, 0.5 mg/mL proteinase K and 5 mM EDTA at 55°C for 1 h. After treatment of the cells with phenol : choloroform : isoamyl alcohol (25 : 24 : 1, vol/vol) and chloroform : isoamyl alcohol (24 : 1, vol/vol) mixtures, DNA was precipitated with 3M sodium acetate (pH 5.2) and absolute ethanol. After washing with 10 mM phosphate buffer (pH 7.4) DNA was dried and re-suspended overnight at 37°C with 1 mM Tris–EDTA buffer (pH 7.0) containing 100 μg/mL RNase A [17].

The extracted DNA was treated with the sample fractions BC-1 and BC-3 (200 μg/mL) at 37°C for 12 h. The control untreated DNA and DNA treated with the BC-1 and BC-3 were subjected to agarose gel electrophoresis. The DNA fragmentation patterns were observed.

Agarose gel electrophoresis. Agarose gel electrophoresis was performed according to available standard protocol of GENEi teaching kit (India). After electrophoresis, the bands were observed in UV light, gel pictures were captured, saved and analyzed using Alphaimager software (USA).

RESULTS AND DISCUSSION

In an attempt to find novel bacteria with antibiotic production potential and other pharmacological activities, 2 bacteria were isolated from soil samples collected in Shimoga region, Karnataka (India) and identified as *B. cereus* and *B. pumilus* with potent antibacterial and antioxidant activities. *B. cereus* has been reported to produce enterotoxins [16], antibacterial [17] and antifungal compounds [18]. The literature survey suggests that some *Bacillus* species have shown anticancer activities. For example, a marine *Bacillus* SW31 has shown growth inhibition and apoptosis of head and neck cancer cells [19]. The cytotoxicity results of this study revealed that *B. cereus* metabolites have compounds with anticancer properties.

Cytotoxicity action (CTC_{50}) of various *B. cereus* (BC-1 to BC-5) and *B. pumilus* (BP-1 to BP-5) sample fractions on different normal and cancer cell lines

Sample fraction	CTC_{50}, μg/mL		
	HepG2	Chang liver	Hep2
BC-1	225.4	659.3	152.2
BC-2	218.5	207.5	1050.2
BC-3	228.3	351.8	282.2
BC-4	147.0	104.8	97.9
BC-5	323.3	223.5	715.3
BP-1	237.4	52.7	282.2
BP-2	209.1	170.0	109.7
BP-3	200.1	258.0	159.9
BP-4	179.0	103.1	54.6
BP-5	350.3	253.6	310.2

Among the studied samples, the fraction BC-1 showed CTC_{50} (cytotoxicity 50%) value of 225.4 μg/mL against HepG2, 152.2 μg/mL against Hep2 and 659.3 μg/mL against Chang liver cells (table). This result demonstrated that fraction BC-1 was more specific towards cancer cells in comparison with normal cells than fraction BC-3. All other fractions obtained from *B. cereus* (BC-2, BC-4 and BC-5) and *B. pumilus* (from BP1 to BP-5) revealed cytotoxicity towards both normal as well as cancer cells, and were not specific against cancer cells. Taking into account this result, BC-1 and BC-3 fractions were taken up for further anticancer studies using nuclear staining method. The action of the metabolites from *B. cereus* demonstrating cytotoxic and apoptotic effect was comparable with that from *Bacillus vallismortis* reported by Jeong et al. [23]. The cancer HepG2 and Hep2 cell lines used in the present work differed from the similar earlier studies. Cytotoxicity studies have confirmed that sample fractions BC-1 and BC-3 were

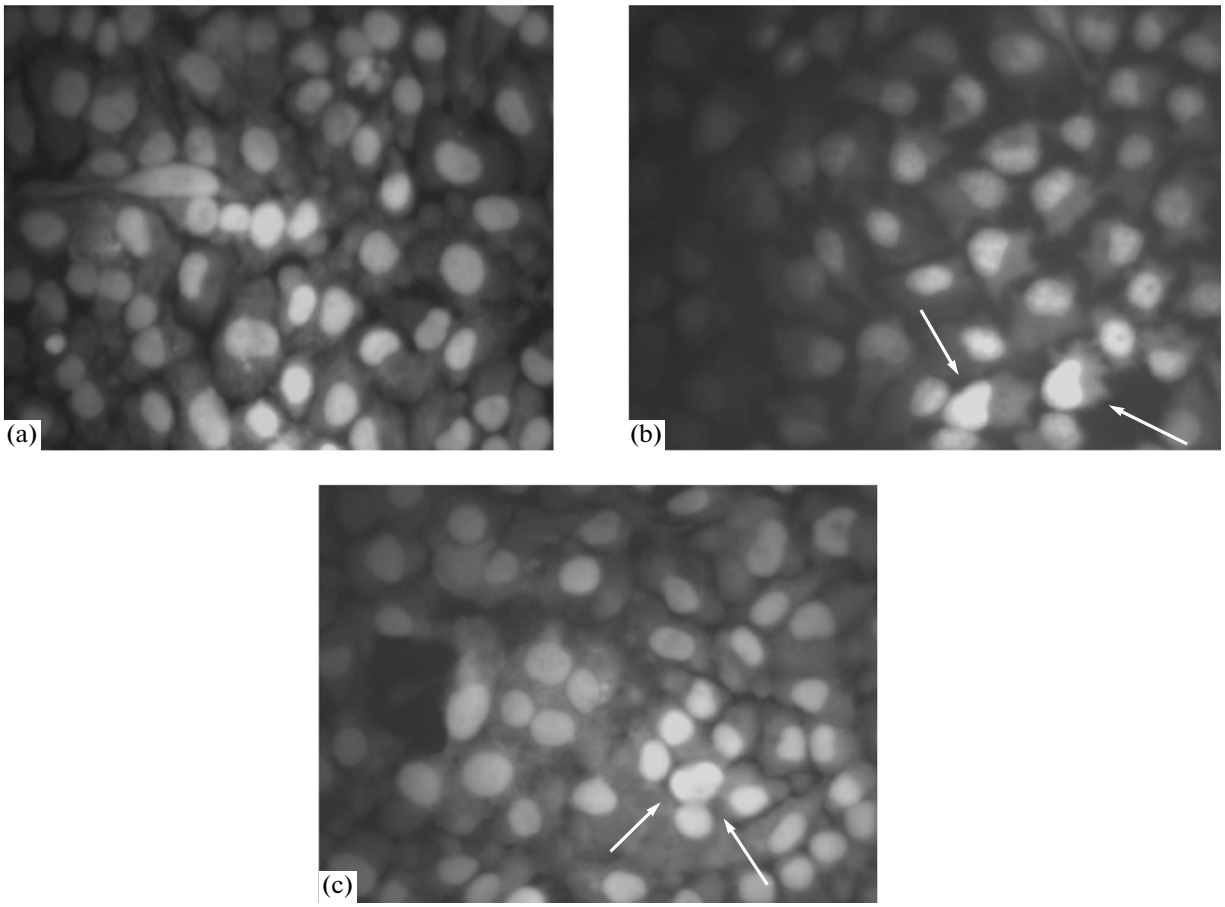

Fig. 2. Nuclear staining of HepG2 cells using acridine orange under fluorescent microscope. a—control cells; b—apoptotic cell morphology of cells treated with fraction BC-1 (200 μg/mL); c—apoptotic cell morphology of cells treated with fraction BC-3 (200 μg/ mL). Arrows indicate the membrane blebbing.

toxic towards cancer cells. Some of the important antitumor compounds used for chemotherapy antibiotics are produced by microorganisms [24–25]. Many bacteria such as *Clostridium*, *Bifidobacterium*, *Salmonella*, *Mycobacterium*, *Bacillus* and *Listeria* have been reported the ability to selective action against target cancer cells by grooving in the hypoxic core regions of solid tumors [26]. Not only live bacteria but also products derived from microorganisms have been tested and some of them are successfully used as anticancer agents. The mode of action is connected with the production of cytotoxic factors, enzymes, antibiotics and other secondary metabolites. SSL proteins produced by *Staphylococcus aureus* and capable to bind "G-protein" receptors which are overexpressed in cancer cells; enzyme called "Ma–ADI" inhibiting tumor growth obtained from *Mycoplasma arginini* and "epothilones", a cytotoxic metabolites with anticancer activity secreted by *Sporangium cellulosum* are a few examples [27–28].

From the results obtained by nuclear morphology studies, it was evident that sample fractions BC-1 and BC-3 showed nuclear morphological changes similar to apoptotic cell morphology in cancerous cell culture HepG2 (Fig. 2). In normal cell culture tested, there was no such nuclear morphological change. This in vitro experiment has proved the selective toxicity of fractions BC-1 and BC-3 against cancer cells. Nuclear staining studies have showed that sample fractions induced apoptosis cell death in liver cancer cells.

The promising result of nuclear staining was the basis for further screening of the same fractions by DNA fragmentation analysis. It was shown that the control DNA had intact band but DNA treated with BC-1 and BC-3 fractions revealed damaged fragmentation patterns (Fig. 3). DNA fragmentation studies have further confirmed that sample fractions caused damage of the cancer cell DNA.

Bacillus thuringiensis, one of the closely related species to *B. cereus*, has been reported to produce unique proteinaceous crystalline parasporins during sporulation with potential cytotoxic and anticancer activity against a number of cancer cell lines [29]. The production of crystalline parasporins is the only fact that discriminates between the two taxonomically closely related species, *B. thuringiensis* and *B. cereus* [30]. This study suggests that the compounds respon-

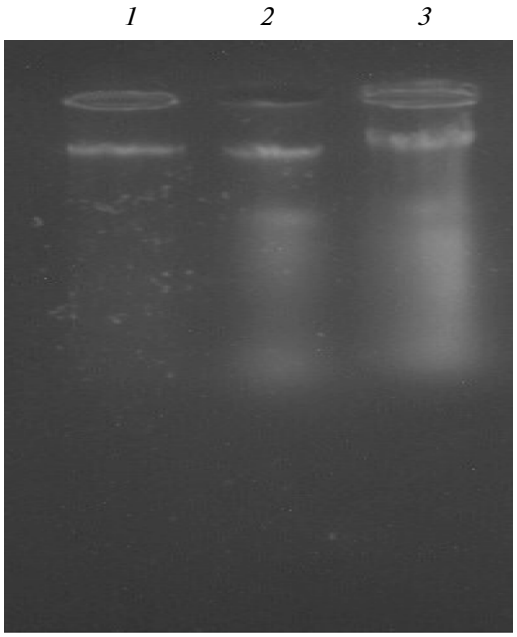

Fig. 3. DNA fragmentation studies of fractions BC-1 and BC-3 in agarose gel electrophoresis. Lane *1*—untreated HepG2 cells; lane *2*—HepG2 cells + cells treated with fraction BC-1 (200 μg/mL; overnight); lane *3*—HepG2 cells + cells treated with fraction BC-3 (200 μg/mL; overnight).

sible for anticancer property of the present isolate *B. cereus* are different from parasporins produced by *B. thuringiensis*. The mass spectroscopy of TLC fractions of BC-1 and BC-3 has revealed that they do not contain proteins (data not shown). Further purification of the TLC fractions and their spectral analysis may provide a lead molecule which can be taken up for in vivo activity and pre-clinical anticancer studies.

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